FOR THE RECORD

Mycobacterium tuberculosis serine/threonine kinases PknB, PknD, PknE, and PknF phosphorylate multiple FHA domains

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Abstract

The physiologic roles and the substrates of the *Mycobacterium tuberculosis* (*Mtb*) serine/threonine kinases are largely unknown. Here, we report six novel interactions of PknB, PknD, PknE, and PknF with the Forkhead-Associated (FHA) domains of Rv0020c and the putative ABC transporter Rv1747. Purified PknB and PknF kinase domains phosphorylated multiple FHA-domain proteins in vitro. Although they remain to be verified in vivo, these reactions suggest a web of interactions between STPKs and FHA domains.

Keywords: FHA domain; protein phosphorylation; serine/threonine protein kinase; signaling

Protein kinases transmit signals through reversible phosphorylation and provide many targets for therapeutic intervention. Genomic analysis of *Mtb* revealed 11 predicted eukaryotic-like serine/threonine protein kinases (STPKs), nine of which are predicted transmembrane receptors (Cole et al. 1998; Av-Gay and Everett 2000). Although initial studies have established roles for STPKs in prokaryotic development, stress responses, and host-pathogen interactions (Av-Gay and Everett 2000), the upstream activators, downstream substrates, effectors, and other binding partners of the STPKs are only beginning to be identified.

Only two putative heterologous in vitro substrates of *Mtb* STPKs have been reported to date: EmbR and Rv1747 (Molle et al. 2003, 2004). EmbR is a transcription factor involved in regulating the synthesis of the cell wall component arabinogalactan (Belanger et al. 1996). EmbR and PknH, the STPK that phosphorylates EmbR, are encoded in the same operon. This interaction and gene organization recapitulate the functional interaction between AfsK and AfsR in *Streptomyces* species first demonstrated in vitro and in vivo by Horinouchi and coworkers (Umeyama et al. 2002). Rv1747 is a predicted ABC transporter that is phosphorylated in vitro by the STPK PknF, which also is encoded in the same operon as its substrate (Molle et al. 2004).

Both of these substrates contain Forkhead-Associated (FHA) domains. FHA domains are ubiquitous phosphothreonine-peptide recognition motifs that play diverse roles in STPK signal transduction in eukaryotes (Durocher and Jackson 2002; Pallen et al. 2002). Rv1747 contains two predicted FHA domains, FHA-A and FHA-B, both of which are necessary for Rv1747 phosphorylation by PknF (Molle et al. 2004). These findings support an emerging model that STPK autophosphorylation, in addition to activating the kinase domains, creates binding sites for substrate proteins containing FHA domains that recognize specific STPK phosphorylation states (Molle et al. 2003, 2004). Moreover, the binding of FHA domains to coexpressed STPKs has given the impression of relatively simple, linear signaling pathways.

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We tested these ideas by assaying for interactions between three of the seven *Mtb* FHA domains (one in Rv0020c and two in Rv1747) and purified, autophosphorylated STPK domains from PknB, PknD, PknE, and PknF. Rv0020c is coexpressed with the STPKs PknA and PknB, and the genes for Rv1747 and PknE, although not coexpressed, are located nearby in the genome. We identified six novel in vitro interactions between STPKs and FHA domains. Contrary to expectations based on the hypothesis that STPKs only bind FHA domains encoded nearby in the genome, the STPKs interacted with FHA domains encoded in different operons. Moreover, we found that the FHA domains themselves can be kinase substrates. These data suggest that STPK-mediated signaling in *Mtb* may be more complex than expected, involving multiple kinase targets in different signaling pathways.

Results and Discussion

Each STPK domain phosphorylated a distinct set of FHA-domain constructs (Fig. 1; Table 1). The PknD and PknE kinase domains, for example, phosphorylated only the Rv1747 FHA-A domain. In contrast, PknB phosphorylated both FHA domains of Rv1747, and PknF phosphorylated all three FHA domains. PknB phosphorylation of the FHA domain of Rv0020c, which is expressed from the same operon as PknB, was not detected. In contrast, PknB efficiently phosphorylated full-length Rv0020c (Fig. 2). To test whether this interaction was FHA-domain dependent, we individually changed Rv0020c Arg459 and Ser473 to Ala. These residues are homologous to the conserved Arg70 and Ser85 in the Rad53 FHA1 domain that have been shown to be critical for phosphopeptide recognition (Durocher et al. 2000). Both mutations abolished phosphorylation of Rv0020c by PknB, demonstrating that Rv0020c phosphorylation depends on the FHA–phosphopeptide interaction (Fig. 2).

All four kinase domains phosphorylated the Rv1747 FHA-GST fusion proteins on the FHA domains, as shown by separating the GST tag from the FHA domain using thrombin (Fig. 1). Although PknF binding to the tandem FHA domains of Rv1747 has been reported, phosphorylation of the FHA domains was not assayed previously. In contrast to the previous finding that both Rv1747 FHA domains are required for PknF binding (Molle et al. 2004), the separate FHA domains A and B were phosphorylated individually (Fig. 1). GST fusion proteins have been shown to form dimers, however, raising the possibility that the Rv1747 FHA-GST fusion proteins mimic the tandem arrangement of these domains in Rv1747. This suggestion is consistent with the observation that efficient phosphorylation of FHA-B by PknF (as well as FHA-A and FHA-B by PknB)

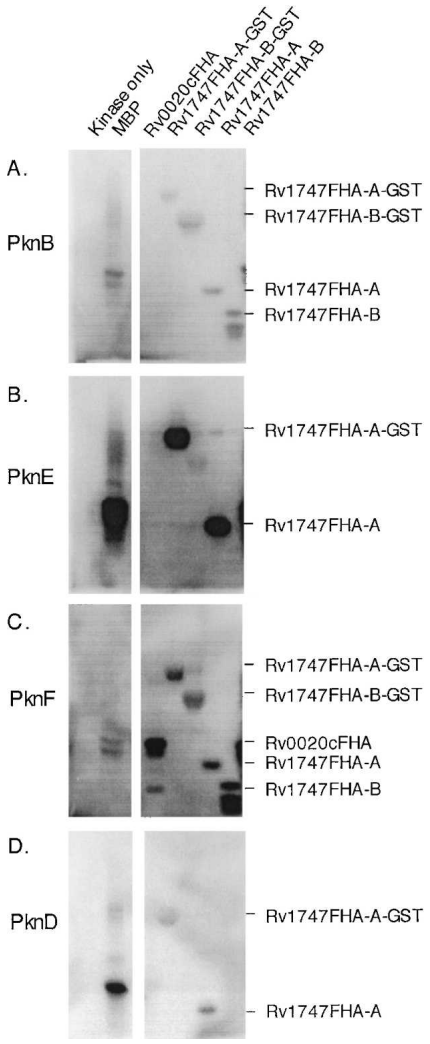

Figure 1. Specific phosphorylation of FHA-domain constructs by *Mtb* STPKs in vitro. The STPK domains of (*A*) PknB, (*B*) PknE, (*C*) PknF, and (*D*) PknD were incubated in the presence of [γ-^{32}P]ATP alone (lane *1*) or with myelin basic protein (MBP, a noncognate substrate; lane *2*), Rv0020cFHA (lane *3*), GST-Rv1747FHA-A (lane *4*), or GST-Rv1747FHA-B (lane *5*). (Lanes *6*,*7*) The GST-Rv1747FHA constructs after treatment with thrombin to release the FHA domain from the GST tag. Samples were separated by SDS-PAGE and visualized by autoradiography.

was observed only in the GST-fusion protein, but not if the FHA domains were cleaved from the GST tag before incubation with kinase (data not shown). In contrast, PknE phosphorylated Rv1747 FHA-A equally well in the presence and absence of the GST tag (data not shown).

FHA domains generally bind phosphothreonine residues in specific peptide or protein contexts. Here, we found a specific set of interactions between three *Mtb* FHA-domains and four STPKs (Table 1). Mutations in the FHA domain of Rv0020c abolished phosphorylation by PknB, suggesting that the binding specificity of the

Table 1. *The* Mtb *STPK domains phosphorylate a specific subset of FHA-domain proteins*

	FHA domains			Full length
	Rv0020c	GST-Rv1747A	GST-Rv1747B	Rv0020c
PknB	–	+	+	+
PknD	–	+/–	–	ND
PknE	–	+	–	ND
PknF	+	+	+	ND

(ND) Not determined.

FHA domains mediates these interactions. Consistent with this conclusion, associations of PknH with the EmbR FHA domain and PknF with the tandem FHA domains of Rv1747 were shown previously to have a similar sensitivity to FHA-domain mutations (Molle et al. 2003, 2004). These results indicate that each FHA-domain protein interacts with a subset of STPKs. This specificity implies that the STPKs mediate diverse signaling pathways rather than triggering a common set of cellular responses.

In contrast to both of the previously reported interactions between FHA domain-containing proteins and coexpressed STPKs, our data demonstrate interactions between STPKs and FHA domains from different operons (Table 1). These data suggest that functional signaling units may involve gene products encoded in diverse locations in the genome. Moreover, the interactions of Rv1747 with multiple STPKs suggest that this ABC transporter may be regulated by multiple signals. If this STPK cross-talk occurs in vivo, the signaling pathways in *Mtb* may be more complex than reported to date. Such general complexity in bacterial STPK signaling is emphasized by the recent findings that three different *Streptomyces* STPKs phosphorylate the transcription factor AfsR in vitro, and at least two of these affect the activity of AfsR in vivo (Sawai et al. 2004). The complexity of signaling networks also is supported by the sequence conservation of the putative peptide substrate-binding groove in orthologs of PknB, which suggests that additional substrates are likely to be recognized by a second, FHA-independent mechanism involving direct binding to surface features of the STPKs (Young et al. 2003).

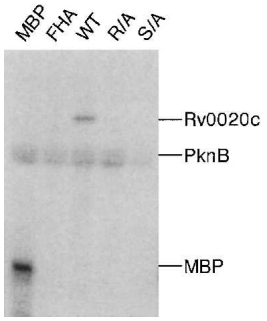

Figure 2. Phosphorylation by PknB requires full-length Rv0020c containing a functional FHA domain. The PknB kinase domain was incubated in the presence of [γ-^{32}P]ATP with substrate proteins MBP (lane *1*), Rv0020c FHA domain (lane *2*), full-length Rv0020c (lane *3*), Rv0020c Arg459Ala (lane *4*), and Rv0020c Ser473Ala (lane *5*). The PknB intracellular domain phosphorylated full-length Rv0020c, but not the FHA domain alone. Mutations in the predicted phosphopeptide recognition site within the FHA domain abolished Rv0020c phosphorylation.

Phosphorylation of the FHA domains themselves (Fig. 1) was unexpected. Although these reactions proceeded efficiently and specifically in vitro, demonstrating their physiologic relevance in vivo remains a significant challenge. The FHA domains might be phosphorylated, for example, merely due to their proximity to the kinase domain upon binding phosphothreonine on the kinase. The lack of phosphorylation of the GST segment of FHA-GST fusion proteins and of the isolated Rv0020c FHA domain, however, indicates that proximity to the kinase domain alone is not sufficient for phosphorylation. Instead, phosphorylation of the FHA domains may serve a physiologic purpose, possibly by modulating target affinity or by providing binding sites for other FHA domains. The in vitro interactions described here set the stage to explore the interactions of *Mtb* FHA-domain proteins and STPKs in vivo.

Materials and methods

Recombinant proteins and kinase assays

To identify substrates of the STPKs, we expressed and purified the complete intracellular segments containing the kinase domains of PknB, PknE, and PknF or the kinase domain only of PknD (Table 2). The His_6-tagged STPK domains were expressed and purified as described (Young et al. 2003)

Table 2. *Protein constructs used in this study*

Construct	Sequence
PknB	His_6-Rv0014c (aa 1–330)
PknD	Rv0931c (aa1–290)-His_6
PknE	His_6-Rv1743 (aa 1–334)
PknF	His_6-Rv1746 (aa 1–301)
Rv0020c	His_6-Rv0020c
Rv0020c R/A	His_6-Rv0020c Arg459Ala
Rv0020c S/A	His_6-Rv0020c Ser473Ala
Rv0020c FHA	His_6-Rv0020c (aa 389–525)
Rv1747 FHA-A	GlySer-Rv1747 (aa 3–116)
Rv1747 FHA-B	GlySer-Rv1747 (aa 203–281)
Rv1747 FHA-A-GST	GST tag-Rv1747 (aa 3–116)
Rv1747 FHA-B-GST	GST tag-Rv1747 (aa 203–281)

using immobilized metal-affinity chromatography and size-exclusion chromatography. Full-length Rv0020c and the Rv0020c FHA domain were expressed and purified using a similar strategy. The Rv0020c Arg459Ala and Ser473Ala mutations were introduced using the QuikChange method (Stratagene). The individual FHA domains A and B of Rv1747 were expressed as GST fusion proteins containing an intervening thrombin cleavage site and purified using Glutathione Sepharose (Amersham Biosciences) and size exclusion chromatography.

Kinase reactions (Young et al. 2003) were carried out in 20 mM PIPES buffer (pH 7.5), 100 nM kinase (200 nM for Rv0020c phosphorylation), 50 μM substrate (5 μM for full-length Rv0020c), 250 μM $MnCl_2$, 75 mM NaCl, 250 μM ATP, and 5 μCi [γ-^{32}P]ATP (MP Biomedicals). The reactions were incubated for 15 min at room temperature and stopped by adding SDS-PAGE sample buffer. The samples were separated by SDS-PAGE, the gels were dried, and the γ-^{32}P incorporation was visualized by autoradiography. The heterologous kinase substrate myelin basic protein (MBP) served as a control to test the activity of the recombinant STPKs.

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